IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Albrecht t al.	
Serial No.:	09/748,642	Examiner: J. Shultz
Filed:	December 22, 2000	Art Unit: 1635
For:	INHIBITION OF CELLULAR PROTEASES	

DECLARATION OF THOMAS ALBRECHT AND ZHENPING CHEN UNDER 37 C.F.R. ' 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

We, THOMAS ALBRECHT AND ZHENPING CHEN, declare:

1. I, Thomas Albrecht received a Ph.D. in 1973 from the Pennsylvania State University College of Medicine in the fields of Microbiology and Biochemistry.

2. From 1986 to this date, I was employed as a Professor at The University of Texas Medical Branch.

3. I, Zhenping Chen received a M.D. in 1982 from the Zhongshan Medical College (which is now called Zhongshan University) and an M.S. in 1988 from Sun Yat-sen University of Medical Sciences (which is now called Zhongshan University)in the field of Oncology.

4. From 1997 to 2006, I was employed as a post-doctoral research associate at the Dept. of Microbiology and Immunology, the University of Texas Medical Branch.

5. I am currently employed as a post-doctoral fellow in the Dept. of Otolaryngology, the University of Texas Medical Branch.

6. We are the inventors of the subject matter claimed in the above-identified patent application.

7. In the above-identified patent application, in an Office Action dated October 5, 2006, at page 2-3, the U.S. Patent and Trademark Office (PTO) rejected claims 6, 7, 14 and 15 under 35 U.S.C. '112(first paragraph) for lack of enablement because:

> [t]he specification as filed does not provide any guidance or examples that would enable a skilled artisan to use the disclosed compounds or methods of using said compounds in vivo, since the prior art is silent on the use of such inhibitors in vivo.

The comments set forth below are intended to demonstrate that calpain inhibitors E64D and Z-Leu-Leu-H are cell permeable and, thus, able to be used in *in vivo* methods.

8. As shown in a paper co-authored by the undersigned inventors, Chen et al., A Degradation of $p21^{cip1}$ in Cells Productively Infected with Human Cytomegalovirus@, J. Virol. 75(8):3613-625(2001)(attached hereto), calpain inhibitors E63d and Z-Leu-Leu-H are cell permeable. (See Page 3617, first paragraph).

9. Further, online catalogs carrying calpain inhibitors E64d and Z-Leu-Leu-H, such as Calbiochem and Peptides International (print out of relevant pages attached hereto) indicate that these calpain inhibitors are cell permeable.

10. Further, as shown in Swapan et al., A Cell Death in Spinal Cord Injury (SCI) Requires de Novo Protein Synthesis@, Annals N.Y. Academy of Sciences 939:436-49 (2001)(abstract), calpain inhibitors, such as E64d, have been used in animal models to treat spinal cord injuries. Results indicate that calpain

activity was almost undetectable in the E64d treated rats.

11. In addition, in the Office Action dated October dated October 5, 2006, at page 3-4, the PTO rejected claims 6 and 7 under 35 U.S.C. '102(a) as anticipated by Chen et al., Proceedings of the American Association for Cancer Research Annual Meeting, 40:447-48(1999)(AChen@). However, Chen describes the work of the inventors.

12. Listed co-author E. Knutson was a technician working in the lab of the inventors and performed experiments under the supervision, direction and control of the inventors.

13. Listed co-author S. Liu was a post-doc fellow working in the lab of the inventors and performed experiments under the supervision, direction and control of the inventors.

14. Listed co-author A. Kurosky was a Professor in the Department of Human Biological Chemistry and Genetics at the University of Texas Medical Branch. Kurosky was consulted to review the findings of the inventors, such findings which were under the direction and control of the inventors

15. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

5 March 2007 Thomas Albrecht
Date Thomas Albrecht

5 March 2007 Zhenping Chen
Date Zhenping Chen

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Degradation of $p21^{cip1}$ in Cells Productively Infected with Human Cytomegalovirus

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Human cytomegalovirus (HCMV) stimulates arrested cells to enter the cell cycle by activating cyclin-dependent kinases (Cdks), notably Cdk2. Several mechanisms are involved in the activation of Cdk2. HCMV causes a substantial increase in the abundance of cyclin E and stimulates translocation of Cdk2 from the cytoplasm to the nucleus. Further, the abundance of the Cdk inhibitors (CKIs) $p21^{cip1/waf1}$ ($p21^{cip1}$) and $p27^{kip1}$ is substantially reduced. The activity of cyclin E/Cdk2 increases as levels of CKIs, particularly $p21^{cip1}$, fall. We have previously shown that these phenomena contribute to priming the cell for efficient replication of HCMV. In this study, the mechanisms responsible for the decrease in $p21^{cip1}$ levels after HCMV infection were investigated by measuring $p21^{cip1}$ RNA and protein levels in permissive human lung (LU) fibroblasts after HCMV infection. Northern blot analysis revealed that $p21^{cip1}$ RNA levels increased briefly at 3 h after HCMV infection and then decreased to their nadir at 24 h; thereafter, RNA levels increased to about 60% of the preinfection level. Western blot analysis demonstrated that the relative abundance of $p21^{cip1}$ protein roughly paralleled the observed changes in initial RNA levels; however, the final levels of protein were much lower than preinfection levels. After a transient increase at 3 h postinfection, $p21^{cip1}$ abundance declined sharply over the next 24 h and remained at a very low level through 96 h postinfection. The disparity between $p21^{cip1}$ RNA and protein levels suggested that the degradation of $p21^{cip1}$ might be affected in HCMV-infected cells. Treatment of HCMV-infected cells with MG132, an inhibitor of proteasome-mediated proteolysis, provided substantial protection of $p21^{cip1}$ in mock-infected cells, but MG132 was much less effective in protecting $p21^{cip1}$ in HCMV-infected cells. The addition of E64d or Z-Leu-Leu-H, each an inhibitor of calpain activity, to HCMV-infected cells substantially increased the abundance of $p21^{cip1}$ in a concentration-dependent manner. To verify that $p21^{cip1}$ was a substrate for calpain, purified recombinant $p21^{cip1}$ was incubated with either m-calpain or μ-calpain, which resulted in rapid proteolysis of $p21^{cip1}$. E64d inhibited the proteolysis of $p21^{cip1}$ catalyzed by either m-calpain or μ-calpain. Direct measurement of calpain activity in HCMV-infected LU cells indicated that HCMV infection induced a substantial and sustained increase in calpain activity, although there was no change in the abundance of either m- or μ-calpain or the endogenous calpain inhibitor calpastatin. The observed increase of calpain activity was consistent with the increases in intracellular free Ca^{2+} and phospholipid degradation in HCMV-infected LU cells reported previously from our laboratory. Considered together, these results suggest that the increase in calpain activity observed following HCMV infection contributes significantly to the reduction of $p21^{cip1}$ levels and the resultant cell cycle progression.

Human cytomegalovirus (HCMV) infection is widespread among human populations, primarily as a subclinical persistent infection. Furthermore, HCMV infection is a majsor cause of morbidity and mortality in several well-studied risk groups. These include congenitally infected infants and individuals with compromised immune systems, particularly after human immunodeficiency virus infection or immunosuppressive therapy for tissue transplantation (for reviews, see references 8, 29, 68, and 72). The clinical management of these infections is still problematic. Although several agents with potent antiviral activity for HCMV infection both in vitro and in vivo have been identified, the toxicity associated with the long-term use of these drugs makes clinical management difficult, and drug-resistant strains of HCMV have emerged (for a review, see reference 61). Thus, there continues to be great interest in improving our understanding of the replication of HCMV with a view toward developing more effective approaches to control these infections.

HCMV replication is associated with extensive modifications of cellular metabolism (reviewed in references 4 and 5), leading to a number of physiologic changes and the activation of a large number of cellular genes (91). Initially, HCMV infection induces a series of cellular responses that resemble the immediate-early events observed following activation of serum-arrested cells by serum growth factors (4). These include hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding increased cellular levels of *sn*-1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate (81); increased release of arachidonic acid and its metabolites (1, 2); changes in Ca^{2+} homeostasis, including Ca^{2+} influx, release of Ca^{2+} stores, and an increase in intracellular free Ca^{2+} (58); transcriptional activation of cellular oncogenes c-*fos*, c-*jun*, and c-*myc* (11, 12, 13); and increased activity of the DNA-binding proteins NFκB, AP-1, and CREB (14). The signaling cascade induced by HCMV infection induces a robust mitogenic response, as evidenced by the ability of HCMV to stimulate cell cycle entry by density-arrested cells, which are resistant to stimulation by serum

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growth factors (19). Recent results indicate that productive HCMV infection stimulates cell cycle progression in either serum- or density-arrested cells through late G_1 phase to a point at or near the G_1/S boundary (19, 30, 52). Closely associated with this limited traverse of the cell cycle is an increase in cyclin E/cyclin-dependent kinase 2 (Cdk2) activity, hereafter referred to as E kinase activity, and hyperphosphorylation of pRb, releasing E2F (45). Activation of E2F, together with Myc, leads to an increase in the cellular levels of a large number of genes involved in nucleotide biosynthesis which primes the infected cell for DNA synthesis (5, 7).

Three HCMV-induced events appear to be necessary for activation of E kinase activity: (i) transcriptional activation of cyclin E (17), (ii) translocation of Cdk2 from the cytoplasm to the nucleus (20), and (iii) a substantial decrease in the nuclear levels of the cyclin kinase inhibitors (CKIs) $p21^{cip1/waf1}$ (hereafter $p21^{cip1}$) and $p27^{kip1}$ (19). Activation of E kinase appears to be critical for efficient HCMV replication, since drugs that interfere with the activity of Cdk2 substantially reduce infectious yields (18). The precise mechanisms through which these virus-induced cellular modifications are achieved are poorly understood. Our long-term goal is to better understand the events that prime the cell for HCMV replication. We are especially interested in the mechanism of reduction of cellular levels of $p21^{cip1}$ during HCMV infection, since this event appears to be closely associated with the increase in E kinase activity (19).

$p21^{cip1}$ is a potent inhibitor of Cdks (41, 42, 84) and is a critical p53-induced downstream effector in the growth-suppressive pathway (33). $p21^{cip1}$ binds cyclin-Cdk complexes, thereby inhibiting the activity of Cdks, such as Cdk2, Cdk3, Cdk4, and Cdk6, and consequently inhibiting cell cycle progression. In addition, $p21^{cip1}$ interacts with proliferating cell nuclear antigen (36) and gadd45 (48), affecting their function, e.g., interfering with DNA replication and repair (24, 36, 51, 60, 62, 82, 86). $p21^{cip1}$ may also be involved in p53-mediated apoptosis (39) and in the control of cell senescence (57).

Despite significant advances in our understanding of how $p21^{cip1}$ exerts its biological effects and is transcriptionally regulated, little is known about how the stability of $p21^{cip1}$ is regulated under the physiologic conditions associated with disease and other forms of stress. Nonlysosomal cytoplasmic protease systems have been identified as important regulators of cell cycle progression (26, 28, 35, 59, 70, 71). Two prominent cytoplasmic protease pathways have been identified: the ubiquitin-proteasome pathway and the calpain pathway. Many of the cell cycle regulatory proteins that are degraded at specific points in the cell cycle, e.g., cyclins A, B, and E, are substrates of the ubiquitin-proteasome pathway. It has also been reported that $p21^{cip1}$ is subject to proteolysis by ubiquitin-mediated proteasome degradation (10, 35, 38, 85). In this study, we undertook a series of experiments to investigate the proteolytic degradation of $p21^{cip1}$ in cells productively infected with HCMV. Consistent with previous work, we observed that the ubiquitin-proteasome pathway is the predominant mechanism mediating the proteolysis of $p21^{cip1}$ in mock-infected or noninfected human embryonic lung (LU) cells. In HCMV-infected LU cells, calpain is activated and is the predominant proteolytic mechanism mediating degradation of $p21^{cip1}$. HCMV infection induced a substantial and prolonged increase in calpain activity when measured in intact cells using fluorogenic substrates or in lysates of infected cells by zymography. The time course for the HCMV-induced increase in calpain activity was consistent with the temporal pattern of $p21^{cip1}$ degradation, and inhibitors of calpain-mediated proteolysis interfered with $p21^{cip1}$ degradation, resulting in a substantial increase in its abundance. These findings provide an explanation for the sustained decrease in $p21^{cip1}$ abundance observed in HCMV-infected cells and, accordingly, help to explain the prolonged activation of E kinase in HCMV infection.

MATERIALS AND METHODS

Cell culture and growth arrest. LU cells (3) were propagated in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS) and penicillin (100 U/ml)-streptomycin (100 μg/ml) in a 5% CO_2 in air atmosphere. The cells were density arrested as described previously in detail (19).

Virus stocks and productive infection. The AD169 strain of HCMV was propagated in LU cells as previously described in detail (6). The infectivities of virus stocks, determined by plaque assay (3), typically were between 8.0×10^6 and 4.0×10^7 PFU/ml. LU cells were infected with HCMV at a multiplicity of 5 PFU/cell to provide a uniform infection as described in detail by Bresnahan et al. (19). Virus stocks and cell cultures were routinely examined for mycoplasma and were free from detectable contamination.

RNA isolation. RNA was extracted using Tri Reagent (Molecular Research Center, Inc., Cincinnati, Ohio), which contains phenol and guanidine thiocyanate, according to the manufacturer's protocol (25).

Preparation of probes. A ^{32}P-labeled DNA probe was prepared from plasmid pC-waf1-S (generously provided by B. Vogelstein [33]), which harbors a $p21^{cip1}$ insert. A probe derived from plasmid p5B (16), which contains the cDNA for 18S rRNA, was used to ensure uniform loading of gels. Plasmids were introduced into competent *Escherichia coli* DH5α cells and amplified. DNA fragments comprising $p21^{cip1}$ and 18S cDNA were excised from isolated plasmid DNA by using the the restriction endonucleases *Not*I and *Bam*HI-*Eco*RI, respectively. The probes were labeled using the Megaprime DNA Labelling System (Amersham Pharmacia Biotech, Piscataway, N.J.).

Electrophoresis and Northern hybridization. Northern hybridization was performed as described previously (17). Total cellular RNA (20 μg/lane) was evaluated under denaturing conditions in formaldehyde-agarose gels, containing 1% agarose, 20 mM 3-(*N*-morpholino) propanesulfonic acid, 1 mM EDTA, 8 mM sodium acetate, and 2.2 M formaldehyde. After separation, the RNA was transferred to MSI nylon membranes (Micron Separations, Inc., Westborough, Mass.) for 18 h. The RNA was prehybridized in Rapid-Hyb buffer (Amersham Life Science, Arlington Heights, Ill.) containing denatured salmon sperm DNA (100 μg/ml) at 65°C for 1 h. ^{32}P-labeled $p21^{cip1}$ or 18S probe was added and hybridized for 2 h at 65°C. Membranes were washed twice with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate (SDS) for 15 min at 65°C, once with 1× SSPE–0.1% SDS at 42°C, and twice with 0.1× SSPE–0.1% SDS at 42°C. The probe remaining on the filter was detected by autoradiography (Kodak [Rochester, N.Y.] X-Omat film for 1 to 16 h at −80°C).

Western blots. Polyclonal and monoclonal antibodies for $p21^{cip1}$ were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Antibodies against m-calpain, μ-calpain, and calpastatin were generously provided by R. L. Mellgren (88). Cells were harvested as we described previously (19) by dislodging the cells with a cell lifter in phosphate-buffered saline (PBS). The cells were collected by sedimentation and lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 7.4] containing 150 mM NaCl and 0.5% NP-40, with 1 mM dithiothreitol [DTT], 1 mM $NaVO_3$, 50 mM NaF, and protease inhibitors [1 mM phenylmethylsulfonyl fluoride {PMSF}, 25 μg of trypsin inhibitor/ml, 25 μg of aprotinin/ml, 1 mM benzamide, and 25 μg of pepstatin A/ml] added just before use). Cellular debris was removed by sedimentation, and the supernatant fluids were reserved. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Whole-cell extracts (40 μg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and the polypeptides were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif.) as described previously (19). Antigen-antibody reactions were detected by the enhanced chemiluminescence assay (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Measurement of intracellular calpain activity. Calpain activity was measured using the cell-permeant fluorogenic calpain substrate *tert*-butoxycarbonyl-L-

leucyl-L-methionineamide-7-amino-4-chloromethylcoumarin (Boc-Leu-Met-CMAC; Molecular Probes, Eugene, Oreg.). HCMV- or mock-infected cells were loaded with Boc-Leu-Met-CMAC (10 μM) for 15 min at selected times after infection. Subsequently, the cells were dissociated by trypsinization and resuspended in PBS. After entering cells, Boc-Leu-Met-CMAC is conjugated to thiols, particularly glutathione, becoming impermeant for the plasmalemma. Cleavage of thiol-conjugated Boc-Leu-Met-CMAC by calpain releases thiol-conjugated 7-amino-4-methylcoumarin (AMC), unquenching the fluorescence. Thus, when cleavage is the rate-limiting step, the observed increase in fluorescence is proportional to the activity of calpain (67). Fluorescence intensities of Boc-Leu-Met-CMAC-loaded cells were measured with a SLM 4800S spectrofluorometer. The relative fluorescence intensities were determined by measuring the fluorescence emission values at intervals from 390 to 550 nm, with excitation set at 380 nm. The fluorescence signal was calculated by integrating the area under the peak at 460 nm. The time interval for loading the cells with Boc-Leu-Met-CMAC, harvesting the loaded cells, and measurement of fluorescence was constant for all samples.

Zymography. Casein zymography was performed by the method of Zhao et al. (90). Cells were removed from culture dishes with a cell scraper into cold PBS, collected by sedimentation, and homogenized in freshly made homogenization buffer [20 mM Tris-HCl (pH 7.4) containing 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg of 4-(2-aminoethyl)benzenesulfonly fluoride (AEBSF)/ml, 5 μg of leupeptin/ml and 10 μg of pepstatin A/ml] at 4°C. Cellular debris was removed by sedimentation, and the supernatant fluids were collected, quick-frozen with liquid nitrogen, and maintained at −80°C until needed.

For electrophoretic separation of the target proteins, 100 μg of protein from each sample was mixed with the appropriate amount of 5× sample buffer (pH 6.8), containing 150 mM Tris-HCl, 20% glycerol, 2 mM 2-mercaptoethanol, and 0.004% bromophenol blue, to obtain a 1× sample buffer concentration. The samples were then fractionated in polyacrylamide gels. The separating gel contained 2% casein (wt/vol; sodium salt) copolymerized with 12% acrylamide, 0.32% (wt/vol) bisacrylamide, 375 mM Tris-HCl (pH 8.8), 0.04% ammonium persulfate, and 0.028% (vol/vol) *N,N,N′,N′*-tetramethylethylenediamine (TEMED). The stacking gel contained 4% acrylamide, 0.11% (wt/vol) bisacrylamide, 380 mM Tris-HCl (pH 6.8), 0.04% ammonium persulfate, and 0.028% (vol/vol) TEMED, without casein. The electrophoresis buffer contained 25 mM Tris base, 192 mM glycine, 1 mM EDTA, and 1 mM DTT (pH 8.3). After electrophoresis, the gel was removed and incubated in incubation buffer for 60 min at room temperature with slow agitation and with two changes of buffer. The incubation buffer contained 20 mM Tris-HCl, 10 mM DTT, 3 mM $CaCl_2$ (pH 7.5 for μ-calpain; pH 7.3 for m-calpain). The gel was incubated for an additional 20 to 24 h and then stained with Coomassie brilliant blue.

Cleavage of p21^{cip1} by calpain. Recombinant p21^{cip1} was a gift from J. Wade Harper (41) or Hengming Ke (53). We also prepared the recombinant p21^{cip1} by using the expression plasmid pET-p21, provided by J. Wade Harper (41), according to the published method (32). To evaluate the effect of calpains on p21^{cip1}, 400 ng of purified recombinant p21^{cip1} was incubated with 0.004 U of either pure μ-calpain or m-calpain at 30°C for 30 min in 40 μl of cleavage buffer (25 mM Tris-HCl [pH 7.5] with 100 mM NaCl, 3 mM DTT, and 5 mM $CaCl_2$) (50). EDTA was added to 10 mM to stop the reaction. An equal volume of 2× SDS-PAGE gel-loading buffer (19) was mixed with the digestion mixture, and the mixture was immediately boiled for 5 min. The digestion products were evaluated by Western blot analysis and SDS-PAGE. Antibody preparations used for the detection of p21^{cip1} (SC-397 and SC-6246) were obtained from Santa Cruz Biotechnology.

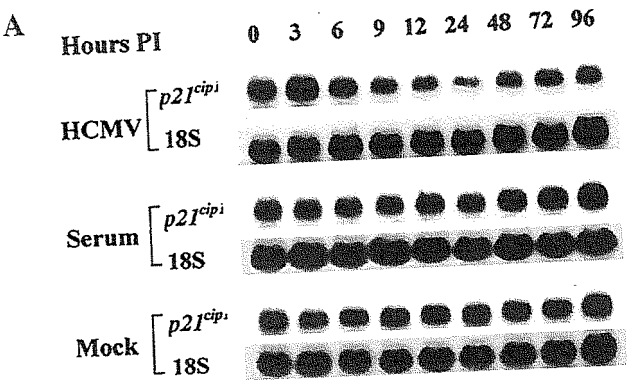
Chemicals. The calpain inhibitors E64d [*trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane] and Z-Leu-Leu-H (carbobenzoxy-L-leucyl-L-leucinal) were purchased from Peptides International, Inc. (Louisville, Ky.). Calpain II (m-calpain), AEBSF, casein, penicillin, streptomycin, diethyl pyrocarbonate, Tris, NaCl, $NaVO_3$, NaF, PMSF, DTT, trypsin inhibitor, aprotinin, benzamide, and pepstatin A were purchased from Sigma (St. Louis, Mo.). Calpain I (μ-calpain) and NP-40 were obtained from Calbiochem (San Diego, Calif.). Boc-Leu-Met-CMAC and CMAC were purchased from Molecular Probes.

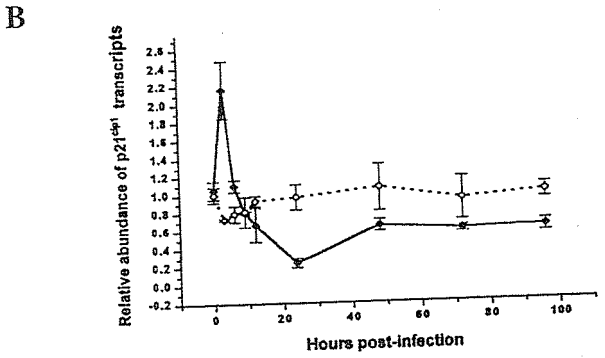
Image processing. All experiments were repeated at least twice, and either data from a representative experiment or means of data from two or more experiments were selected for presentation. Chemiluminescent samples were exposed for intervals that ensured a linear response, as determined by standardization. All radiographic films were analyzed using an AlphaImager 2000 documentation and analysis system (Alpha Innotech Corp.) and the manufacturer's software (AlphaImager 2000, version 3.x). The images were quantified and recorded as tagged image format files, which were then used to prepare the graphic images presented in this report.

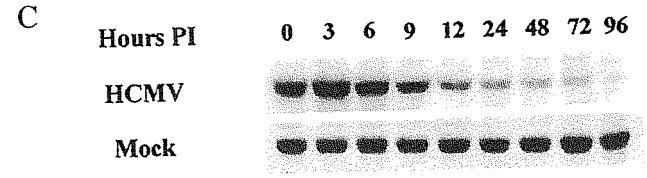
RESULTS

p21^{cip1} RNA and protein levels in HCMV-infected LU cells. Previous studies from our laboratory demonstrated that HCMV infection resulted in a substantial decrease in the abundance of p21^{cip1} protein in LU cells (19). To investigate the mechanism responsible for this drop, p21^{cip1} transcript levels were measured by Northern blot analysis in density-arrested LU cells following stimulation with 10% FBS, HCMV (5 PFU/cell) infection, or mock infection. The results of a representative experiment are shown in Fig. 1A. In density-arrested cells, FBS treatment or mock infection had little effect on p21^{cip1} RNA levels. Following HCMV infection of density-arrested LU cells, p21^{cip1} RNA levels increased briefly at 3 h postinfection (p.i.) and then decreased over the next 21 h p.i., whereupon they began to recover, reaching 60% of their pre-infection level at 96 h p.i. (determined by densitometry [Fig. 1B]). Western blot analysis (Fig. 1C) demonstrated that p21^{cip1} protein abundance was not correlated with the observed changes in RNA levels after 24 h p.i. After an early increase at 3 h p.i., p21^{cip1} protein levels fell dramatically from 3 to 24 h p.i., asymptotically approaching the limits of detection from 48 to 96 h p.i. (Fig. 1D). These findings provide clear evidence of a disparity between p21^{cip1} RNA and protein levels in the HCMV-infected cells after 24 h p.i.

Differential effects of an inhibitor of the ubiquitin-proteasome proteolytic pathway on p21^{cip1} degradation during HCMV infection and mock infection. Since the ubiquitin-proteasome pathway is reported to be responsible for degrading many of the cell cycle regulatory proteins, we investigated whether or not the decrease of p21^{cip1} in HCMV-infected cells was due to a proteasome-mediated mechanism. HCMV (5 PFU/cell)-infected and mock-infected LU cells were treated with MG132, an inhibitor of proteasome-mediated proteolysis (56, 69), at 1 h p.i., and p21^{cip1} abundance was evaluated at 24 h p.i. The results of a representative experiment are shown in Fig. 2A. By 24 h p.i., p21^{cip1} levels were substantially lower in HCMV-infected LU cells (Fig. 2A) than in the control (mock-infected cells). MG132 stabilized p21^{cip1} in mock-infected, density-arrested LU cells but had only a limited effect on stabilization of p21^{cip1} in HCMV-infected cells (Fig. 2). Mock-infected, density-arrested LU cells treated with MG132 (0 to 10 μM) demonstrated a concentration-dependent increase in the abundance of p21^{cip1}. A greater than fivefold increase in p21^{cip1} abundance relative to either the dimethyl sulfoxide (DMSO) control (lane 2) or cells in the absence of any chemical (lane 1) was observed at concentrations of 5 and 10 μM (Fig. 2). In HCMV-infected cells, smaller increases in p21^{cip1} abundance were observed at MG132 concentrations from 2.5 to 10 μM, reaching a maximum 2.5-fold increase at 10 μM. That the p21^{cip1} abundance was less responsive to protection by MG132 in HCMV-infected cells than in mock-infected cells is demonstrated by the significant difference ($P < 0.01$) in the slopes (determined by linear regression analysis) of the concentration effect of MG132 in HCMV-infected (0.800, $P < 0.01$) and mock-infected (1.964, $P < 0.01$) LU cells. These findings suggested that some additional MG132-insensitive pathway(s) may be involved in the proteolysis of p21^{cip1} in HCMV-infected cells. As the unidentified mechanism(s) seemed to be quantitatively more important in p21^{cip1} prote-







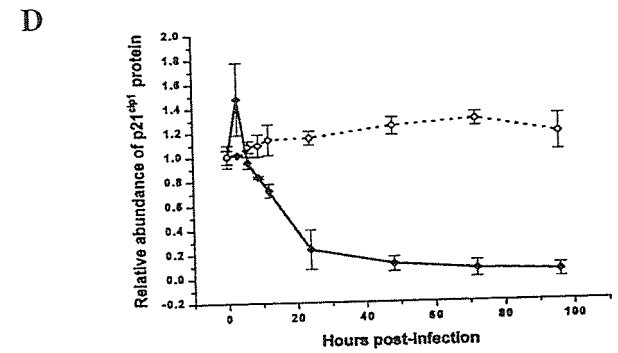
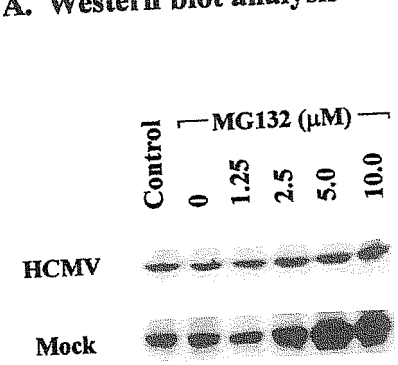


FIG. 1. (A) Northern blot analysis of the effect of HCMV infection (HCMV), serum growth factors (Serum), or mock infection (Mock) on $p21^{cip1}$ RNA transcripts in density-arrested LU cells. The density-arrested cells were infected with HCMV at a multiplicity of 5 PFU/cell, exposed to fresh FBS (10%), or mock infected. RNA was isolated at the times indicated, and 20 μg of RNA from each lysate was applied to the gel. 18s rRNA (18S) was used as a loading standard. (B) Results of the representative experiment illustrated in panel A and of two additional independent experiments, evaluated by densitometric analysis and plotted as the abundance relative to the mock-infected control at 0 h p.i. with standard deviation shown (◆, $p21^{cip1}$ RNA, HCMV-infected cells; ◇, $p21^{cip1}$ RNA, mock-infected cells). (C) Western blot analysis of the abundance of $p21^{cip1}$ after HCMV infection or mock infection. Parallel cultures of density-arrested LU cells were treated as



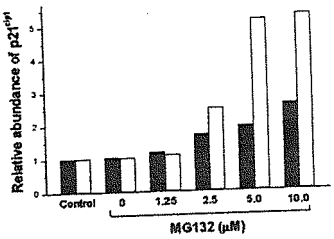


FIG. 2. Effect of MG132, an inhibitor of proteasome activity, on $p21^{cip1}$ abundance in HCMV- or mock-infected, density-arrested LU cells. (A) Results of a representative experiment in which density-arrested LU cells were infected with HCMV (5 PFU/cell) or mock infected and then exposed to the indicated concentrations of MG132 beginning at 24 h p.i. Cell lysates were prepared at 30 h p.i. and 40 μg of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies against $p21^{cip1}$. Control, cells cultured in the absence of MG132 or its solvent, DMSO; 0, DMSO control, consisting of cells treated with the same concentration of DMSO (0.1%) present in the cultural fluids containing the highest concentration of MG132 (10 μM). (B) Change in abundance of $p21^{cip1}$ after MG132 treatment, determined by densitometry of the results illustrated in panel A. The results for mock-infected cells (open bars) were calculated relative to the $p21^{cip1}$ abundance in untreated mock-infected cells; the results for HCMV-infected cells (closed bars) were determined relative to the $p21^{cip1}$ abundance in untreated HCMV-infected cells.

olysis in HCMV-infected cells than was proteasome-mediated degradation, we were interested in identifying the proteolytic mechanism(s) stimulated by HCMV infection.

The calpain inhibitors E64d and Z-Leu-Leu-H protect $p21^{cip1}$ from degradation during HCMV infection. HCMV in-

in the legend to panel A. Whole-cell lysates were prepared at the indicated intervals, and 40 μg of protein from each was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with antibodies against $p21^{cip1}$. The results of a representative experiment are illustrated. (D) The results of the experiment illustrated in panel C and two additional experiments, evaluated by densitometric analysis and plotted as the abundance relative to the mock-infected control at 0 h p.i. with standard deviation shown (◆, $p21^{cip1}$ protein, HCMV-infected cells; ◇, $p21^{cip1}$ protein, mock-infected cells).

fection induces substantial increases in intracellular free Ca^{2+} (58) and in phospholipid degradation (1, 2, 81), raising the possibility that Ca^{2+}-activated neutral proteases (calpains) might be activated by HCMV infection and participate in the proteolysis of p21^{cip1}. Previous work has demonstrated that calpains are able to cleave cell cycle regulatory proteins, such as cyclin D1, cellular oncogene products (e.g., c-Mos, c-Jun, and c-Fos), and p53 (43, 59; for reviews, see references 21 and 28). The possibility that calpain-mediated proteolysis was involved in the degradation of p21^{cip1} in HCMV-infected cells was investigated first by examining the effect of the cell-permeable calpain inhibitors E64d (79) and Z-Leu-Leu-H (69) on p21^{cip1} levels following HCMV infection.

In initial experiments, we measured the effect of E64d (100 μM) on p21^{cip1} levels by Western blot analysis beginning at 48 h p.i., when p21^{cip1} was near its lowest level (Fig. 1) in HCMV-infected (5 PFU/cell), density-arrested LU cells. The abundance of p21^{cip1} was determined at selected intervals for 24 h following treatment with E64d. The results of a representative experiment are shown in Fig. 3. As noted previously, a substantial decrease in p21^{cip1} levels was observed at 48 h p.i. in HCMV-infected cells just prior to treatment with E64d (Fig. 3, compare lane 1 [lysate of mock-infected cells prepared at 0 h p.i.] to lane 2 [lysate of HCMV-infected cells in the absence of E64d prepared at 48 h p.i.]). Except at 3 h after treatment with E64d, when there was about a 1.5-fold increase in p21^{cip1} in the presence of E64d, the calpain inhibitor had little, if any, effect on the abundance of p21^{cip1} in mock-infected, density-arrested LU cells. In contrast, in HCMV-infected cells in the presence of E64d, a substantial accumulation of p21^{cip1} was observed, with a fourfold increase in the abundance of p21^{cip1} in HCMV-infected cells observed after 12 h of E64d treatment. In the absence of E64d, levels of p21^{cip1} in HCMV-infected cells remained at a constant low level. The maximum level of p21^{cip1} in the E64d-treated cells under these conditions was about 84% of that observed at 0 h. These data suggested that an E64d-sensitive proteolytic pathway was induced in HCMV-infected cells and was largely responsible for a substantial decrease in the abundance of p21^{cip1}.

The concentration effect of E64d on p21^{cip1} abundance was examined in HCMV (5 PFU/cell)- and mock-infected, density-arrested LU cells by addition of selected concentrations of E64d at 48 h p.i. Whole-cell extracts were prepared 6 h later and evaluated by Western blot analysis. The results of a representative experiment are shown in Fig. 4. Since E64d had little, if any, effect on p21^{cip1} levels in mock-infected cells even at a concentration of 100 μM, as demonstrated in Fig. 3, the data for mock-infected cells are not illustrated in Fig. 4. In HCMV-infected cells, protection of p21^{cip1} was observed at all concentrations evaluated (6.25 to 100 μM), while the quantitative effect was directly dependent on the concentration of E64d. Cells treated with the highest concentration of E64d (100 μM) demonstrated about 85% of the p21^{cip1} abundance measured in mock-infected cells over the same time period (Fig. 4B, compare the first and last columns).

Protection of p21^{cip1} by Z-Leu-Leu-H was evaluated by the same approach as described above. Z-Leu-Leu-H also protected p21^{cip1} in a concentration-dependent manner in HCMV (5 PFU/cell)-infected, density-arrested LU cells, with some protection provided by all concentrations of Z-Leu-Leu-H

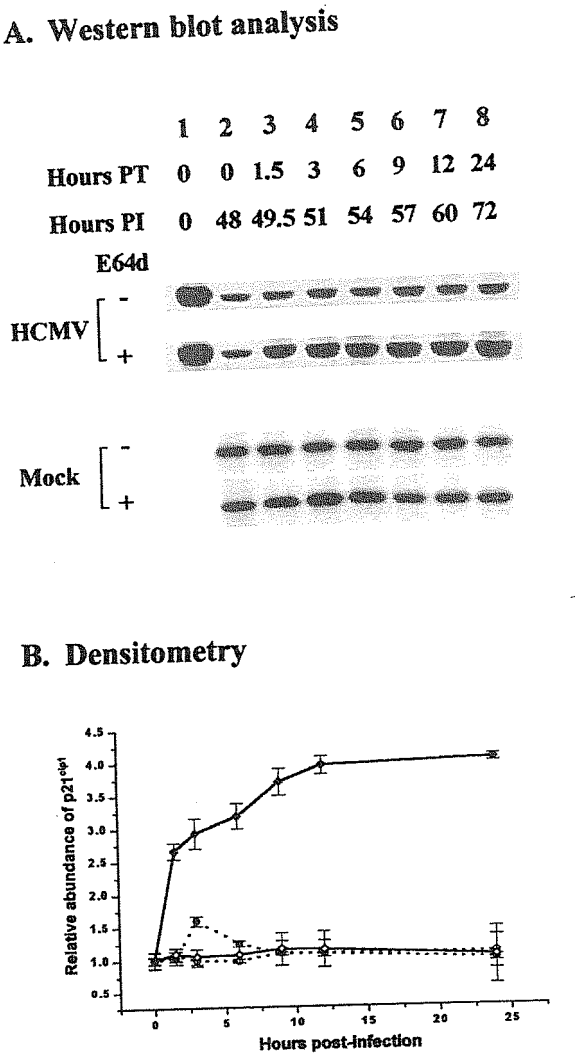


FIG. 3. Cumulative effect of the calpain inhibitor E64d (100 μM) on p21^{cip1} protein levels in HCMV- or mock-infected, density-arrested LU cells. (A) LU cells arrested by contact inhibition as described in Materials and Methods were infected with HCMV (5 PFU/cell) or mock infected and then exposed to E64d beginning at 48 h p.i. At the times indicated, whole-cell lysates were prepared, and 40 μg of protein from each lysate was analyzed by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies against p21^{cip1}. The results of a representative experiment are shown. Hours PT (posttreatment) indicates the duration of E64d exposure. Hours PI indicates the time of harvest after infection. The exposure time for blots of lysates from HCMV-infected cells was about three times longer than that required for blots of lysates from mock-infected cells. Accordingly, to permit a quantitative comparison between the p21^{cip1} abundance at 0 h p.i. and at later times, lane 1 for the HCMV-infected cell lysates was loaded with a lysate from mock-infected cells not treated with E64d. (B) Accumulation of p21^{cip1} after E64d treatment, determined by densitometry of the data illustrated in panel A and from two additional experiments. The results for p21^{cip1} abundance in HCMV- and mock-infected cells with standard deviation shown are plotted relative to the p21^{cip1} abundance at 0 h in the absence of E64d in HCMV- and mock-infected cells, respectively (○, mock-infected cells; ●, mock-infected cells treated with E64d; ◇, HCMV-infected cells; ◆, HCMV-infected cells treated with E64d).

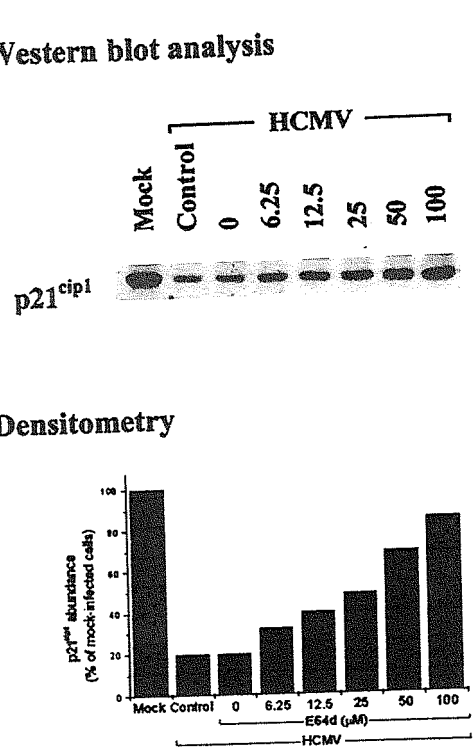


FIG. 4. Effect of E64d concentration on $p21^{cip1}$ abundance in HCMV-infected cells. (A) LU cells were density arrested as described in Materials and Methods, HCMV infected (5 PFU/cell) or mock infected, and at 48 h p.i. treated with the indicated concentrations of E64d. Whole-cell lysates were prepared 6 h later, and 40 μg of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies to $p21^{cip1}$. The results from a representative experiment are shown. Mock, mock-infected cells in the absence of drug; Control, untreated HCMV-infected cells; 0, DMSO control, consisting of HCMV-infected cells treated with the same concentration of DMSO (0.1%) contained in the highest concentration of E64d. (B) Densitometric analysis of the results shown in panel A.

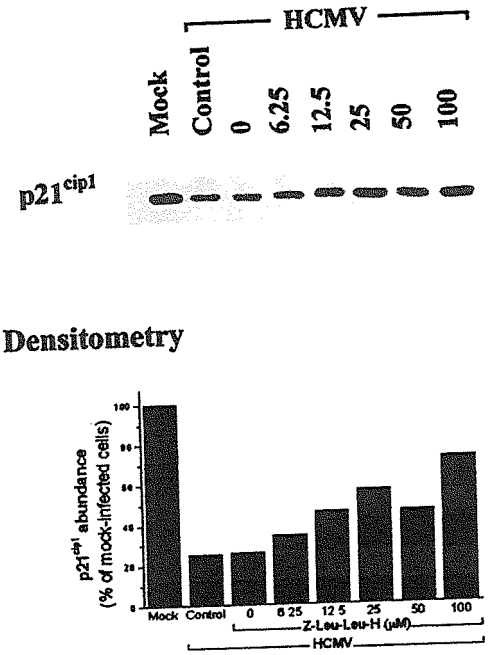


FIG. 5. Effect of Z-Leu-Leu-H concentration on $p21^{cip1}$ abundance in HCMV-infected cells. (A) LU cells were density arrested as described in Materials and Methods, infected with HCMV (5 PFU/cell) or mock infected, and at 48 h p.i. treated with selected concentrations of Z-Leu-Leu-H. Whole-cell lysates were prepared 6 h later, and 40 μg of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies to $p21^{cip1}$. The results illustrated are those from a representative experiment. Mock, Control, and 0 are defined in the legend to Fig. 4. (B) Densitometric analysis of the results illustrated in panel A.

evaluated. The results of a representative experiment are shown in Fig. 5. The levels of protection provided by Z-Leu-Leu-H were similar to those observed with E64d (Fig. 4). A concentration of 100 μM of Z-Leu-Leu-H resulted in detection of about 72% of the mock-infected levels of $p21^{cip1}$ (Fig. 5B, compare the first and last columns), compared to 85% for cells treated with E64d. Z-Leu-Leu-H had little, if any, effect on the abundance of $p21^{cip1}$ in mock-infected cells (data not shown).

Sensitivity of $p21^{cip1}$ degradation to inhibition by E64d during the HCMV replication cycle. To determine the effect of E64d (100 μM) on $p21^{cip1}$ levels throughout the time course of HCMV infection, density-arrested LU cell cultures were either infected with HCMV (5 PFU/cell) or mock infected and treated with E64d up to 12 h before harvest. For cells harvested at 12 h p.i. or before, the cells were treated with E64d from 1 h p.i. to the time of harvest (Fig. 6). E64d had little effect on $p21^{cip1}$ levels in mock-infected cells, as previously noted (Fig. 3), and therefore the results for mock-infected cells are not shown. The results for HCMV-infected cells from a representative experiment are shown in Fig. 6A. In the absence of E64d, $p21^{cip1}$ levels in HCMV-infected cells increased at 3 h and began to drop off by 6 h. By 72 and 96 h p.i., $p21^{cip1}$ was only faintly detectable in the HCMV-infected cells in the absence of E64d. In the presence of E64d, $p21^{cip1}$ levels in HCMV-infected cells were significantly ($P < 0.05$) greater (from 3 h to 96 h p.i., except at 12 and 96 h, when the difference, although greater, was not significant) than the levels observed in the absence of the calpain inhibitor (Fig. 6B). As a result of E64d treatment, $p21^{cip1}$ levels remained at or above the preinfection levels through 9 h p.i. and within 85% of the preinfection levels through 24 h. After 48 h, $p21^{cip1}$ levels in E64d-treated, HCMV-infected cells remained well above the levels in the absence of the calpain inhibitor, but the $p21^{cip1}$ abundance dropped progressively below the preinfection levels. Considered together, these data suggested that calpain-mediated proteolysis contributed substantially to the degradation of $p21^{cip1}$ in HCMV-infected cells from 3 h through the late phase of HCMV infection.

Calpain is activated by HCMV infection. The level of calpain activity is normally very low in unstimulated cells due to the presence of the endogenous calpain inhibitor calpastatin (21). The findings that treatment of HCMV-infected cells with E64d or Z-Leu-Leu-H led to stabilization and accumulation of $p21^{cip1}$ suggested that calpain is activated during HCMV in-

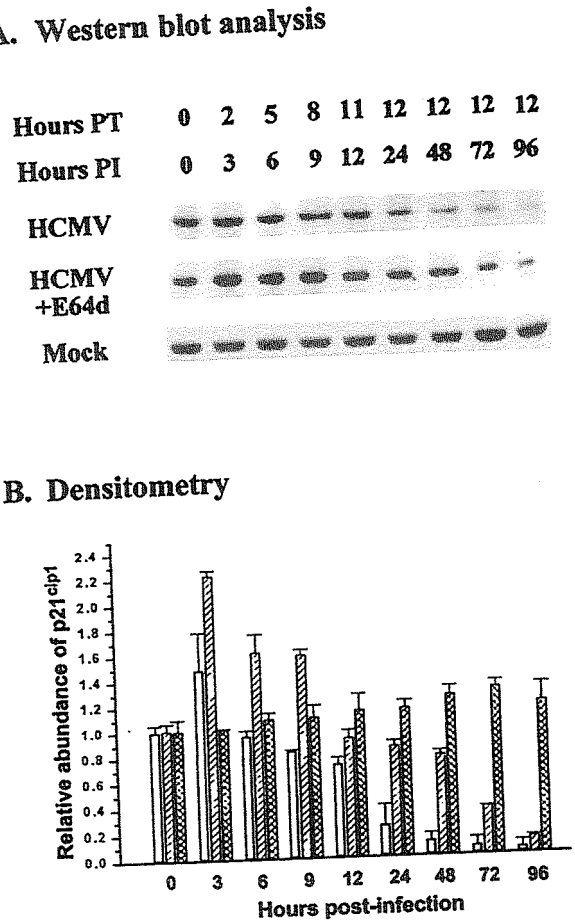


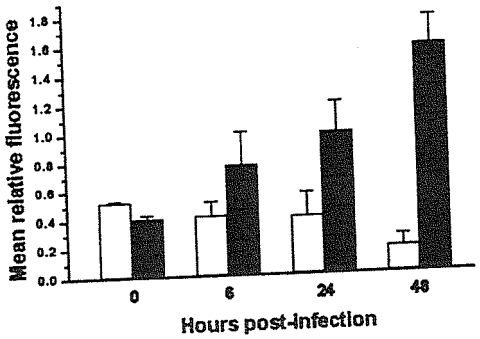
FIG. 6. Time course for the effect of E64d on $p21^{cip1}$ abundance in HCMV-infected, density-arrested cells. (A) LU cells density arrested as described in Materials and Methods were infected with HCMV (5 PFU/cell) or mock infected; at the intervals indicated, beginning 1 h p.i., subsets of the cells were treated with E64d (100 μM). Whole-cell lysates were prepared at the times indicated (up to 12 h posttreatment [PT]), and 40 μg of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies to $p21^{cip1}$. The results of a representative experiment are illustrated. (B) Densitometric analysis of the results shown in panel A and two additional experiments with standard deviation. Open bars, HCMV-infected cells; diagonally striped bars, HCMV-infected cells treated with E64d; cross-hatched bars, mock-infected cells.

fection. To investigate this possibility, we used the fluorogenic calpain substrate Boc-Leu-Met-CMAC to measure calpain activity in density-arrested LU cells (67). Density-arrested LU cells were infected with HCMV (5 PFU/cell) or mock infected and exposed to Boc-Leu-Met-CMAC (10 μM) 15 min before assay. The cells were harvested (see Materials and Methods), and the relative fluorescence was determined. The results (Fig. 7A) demonstrated that fluorescence from the product (AMC) of cleavage of the fluorogenic substrate fell gradually in mock-infected cells during the 48-h course of these experiments. HCMV infection, however, induced a substantial increase in calpain activity. An increase in AMC fluorescence was observed by 6 h p.i. in HCMV-infected cells. After 24 h, the AMC fluorescence in HCMV-infected cells increased to a value about 2.5-fold greater than that observed in mock-infected cells. The relative fluorescence observed in the HCMV-infected cells increased further by 48 h p.i., when it was about eightfold greater than the fluorescence in mock-infected cells. The increases in calpain activity from 24 and 48 h were significant ($P < 0.05$). These data closely reflect the time course for the changes in the abundance of $p21^{cip1}$ following HCMV infection in our earlier work (19) and in this study. In these studies, a decrease in $p21^{cip1}$ levels was first detected at 6 h p.i., and the abundance of $p21^{cip1}$ continued to decrease through 48 h p.i.

Zymography. Since casein is a well-established target of calpain-mediated proteolysis (87), we used casein zymography to verify that calpain was activated in HCMV-infected cells, to determine which calpain isoenzymes were activated, and to extend the time course data for calpain activity. Density-arrested LU cells were infected with HCMV (5 PFU/cell) or mock infected, harvested at times from 0 to 96 h p.i., and evaluated by casein zymography. The results of casein zymography to evaluate cellular proteolysis are illustrated in Fig. 7B. When both of the ubiquitous calpains are activated, μ-calpain is observed as the upper band, while m-calpain is observed at a slightly lower position in the nondenaturing gels (89). The intensity of the bands was related linearly to the amount of protease activity. Since μ- and m-calpain have slightly different pH optima, duplicate gels were incubated at pH 7.5 or 7.3 to evaluate quantitative changes in isoenzyme activity. There was substantial activity of both ubiquitous calpains at each pH shown when both calpains were activated. The results for a representative experiment from this series of experiments (Fig. 7B) demonstrated that calpain activity gradually decreased in mock-infected cells during the 96-h time course of the experiment. In contrast, HCMV infection induced an increase in both μ- and m-calpain activities by 6 to 9 h p.i. (Fig. 7B, part 2). By 24 h, the calpain activity of HCMV-infected cells was about fourfold (μ-calpain) or fivefold (m-calpain) greater than that of mock-infected cells. The enzyme activity continued to increase and reached its maximum level at 72 h p.i. when it was about 23-fold (μ-calpain) or 25-fold (m-calpain) greater than that observed in mock-infected cells. The increases in calpain activities from 12 to 96 h were significant ($P < 0.05$) relative to the preinfection levels. Overall, the changes in calpain activity measured by casein zymography were remarkably similar to those obtained using the fluorogenic calpain substrate Boc-Leu-Met-CMAC in intact cells (Fig. 7A) and indicate that HCMV infection was activating both μ- and m-calpain.

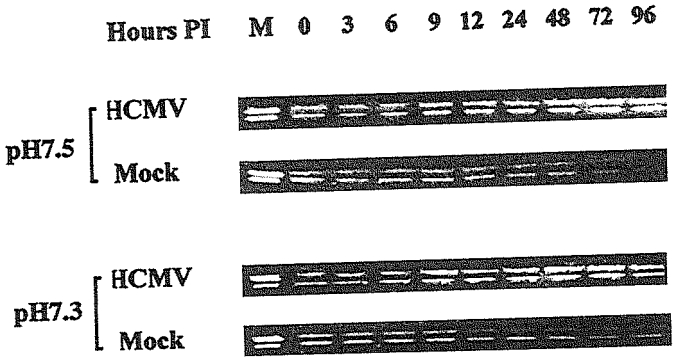
To determine if the increased calpain activity observed in Fig. 7A and B was a result of increased expression of calpains or a decrease in the abundance of calpastatin, the abundance of these proteins was measured by Western blot analysis (Fig. 7C) in cells treated in parallel with those used to measure calpain activity (Fig. 7B). HCMV infection had little, if any, detectable effect on the abundance of μ-calpain or calpastatin through 96 h p.i. m-Calpain levels demonstrated a very slight increase from 6 h to 72 h p.i. over the abundance observed in mock-infected cells, and by 96 h, HCMV- and mock-infected cells demonstrated similar levels of m-calpain. Given the very small difference in the abundance of m-calpain in HCMV- and mock-infected cells, it was questionable if this difference had any significance. Mock infection had little, if any, effect on the

A. Calpain activity-Fluorescence

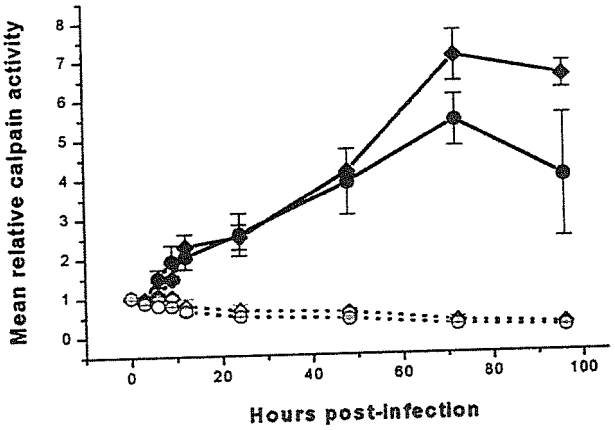


B. Calpain activity-Zymogram

1. Zymogram

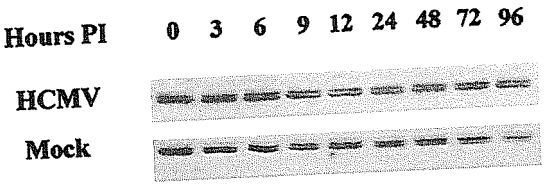


2. Densitometry

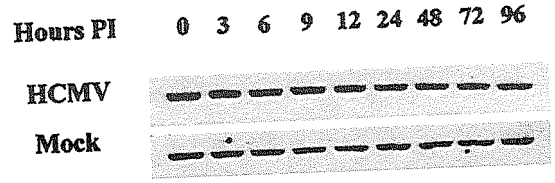


C. Western blot analysis

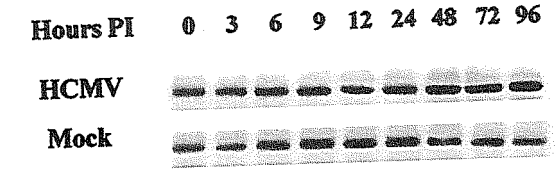
1. m-Calpain



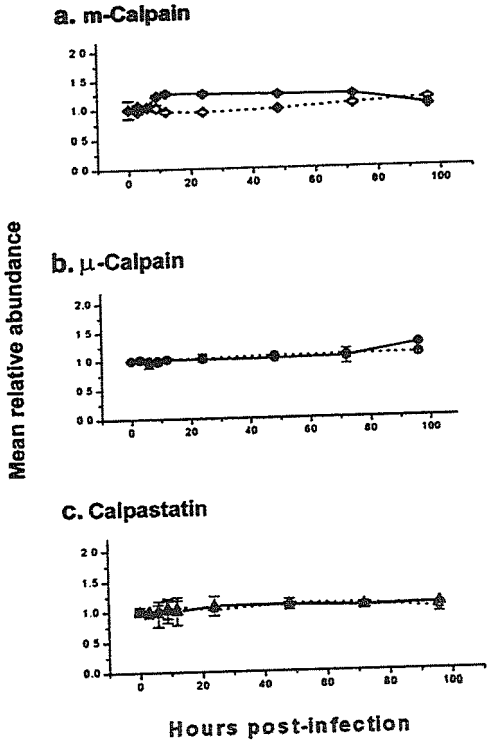
2. μ-Calpain



3. Calpastatin



4. Densitometry



cellular levels of μ-calpain, m-calpain, or calpastatin during the time course of this series of experiments. Thus, it is unlikely that the substantial increase in calpain activity observed in HCMV-infected cells was a result of a decrease in calpastatin abundance or of increases in the abundance of the ubiquitous calpains.

Calpain cleaves p21cip1. Although a number of proteins are now recognized as targets of calpain-mediated proteolysis (21), p21cip1 has previously not been included among them. Accordingly, we were interested in directly testing the sensitivity of p21cip1 to calpain-mediated proteolysis by incubating purified recombinant p21cip1, obtained from J. Wade Harper (41) or Hengming Ke (53), with either purified μ-calpain or m-calpain and evaluating the products by Western blot analysis. We also prepared recombinant p21cip1 by using the expression plasmid pET-p21, provided by J. Wade Harper (32, 41). Incubation of any of the purified p21cip1 preparations with purified μ-calpain or m-calpain resulted in the rapid cleavage of p21cip1, as illustrated with findings for μ-calpain in Fig. 8A. Two prominent p21cip1 fragments were observed after incubation with calpain, consistent with the results from earlier studies indicating that calpain typically cleaves its target proteins at a limited number of sites (for a review, see reference 78). Inclusion of E64d in the digestion inhibited the proteolysis of p21cip1 (Fig. 8A). To investigate if either of the ubiquitous calpains was removing or modifying the availability of the epitope that was the target of the anti-p21cip1 antibody used in the Western blot analysis, we incubated p21cip21 and casein separately with either μ-calpain or m-calpain and examined the proteolytic products by SDS-PAGE. The location of polypeptides in the gels was demonstrated by Coomassie brilliant blue staining. To minimize the loss of small peptides, electrophoresis of proteins applied to these gels was for a period of time shorter than used for Western blot analysis (Fig. 8A). A limited number of cleavage products was observed in the Coomassie blue-stained gels (Fig. 8B) for either casein or p21cip1 incubated with either of the ubiquitous calpains, as had been observed by Western blot analysis (Fig. 8A). Thus, p21cip1 appeared to be a target for proteolysis mediated by either μ- or m-calpain.

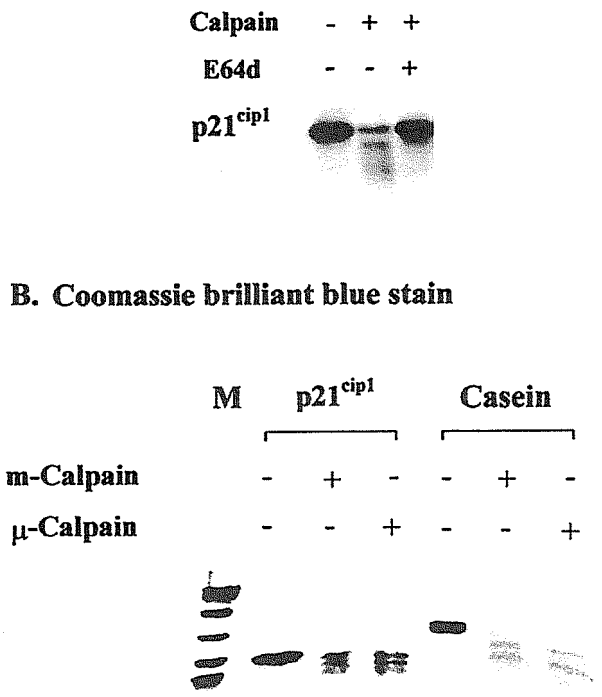


FIG. 8. Cleavage of purified recombinant p21cip1 by purified calpain. (A) p21cip1 (0.4 μg) was incubated with μ-calpain (0.004 U) for 30 min at 30°C in the presence or absence of E64d (100 μM), and the resulting products were analyzed by Western blot analysis as described in Materials and Methods. The data illustrated are from a representative experiment. (B) p21cip1 (4 μg) or casein (3 μg) was incubated with 0.04 U of μ-calpain or m-calpain for 30 min at 30°C, and the products of the digestion were examined by SDS-PAGE and Coomassie brilliant blue staining. M, Rainbow colored protein molecular weight markers of 97.4, 66, 46, 30, 21.5, and 14.3 kDa (Amersham Life Science). The data illustrated are from a representative experiment.

DISCUSSION

Involvement of p21cip1 in cell cycle progression in HCMV-infected cells. Progression through the cell cycle is regulated by mechanisms that are beginning to be understood (31, 73, 74). Induction of cyclin E-associated kinase activity is essential for progression through the G_1 phase of the cell cycle (for a review, see reference 49) and preparation of the cell for DNA

FIG. 7. (A) The time course for calpain activity in HCMV (5 PFU/cell)-infected (solid bars) and mock-infected (open bars) density-arrested cells. Calpain activity was measured by exposing the cells to the cell-permeant fluorogenic calpain substrate Boc-Leu-Met-CMAC (18 μM) for 15 min before the fluorescence intensity was measured for equal numbers of HCMV- and mock-infected cells, using an SLM 4800S spectrofluorometer. Excitation was at 380 nm; emission was at 460 nm. The polarizers were set at 0° and 50°. The data plotted are the means (± standard deviation) of the findings from three independent experiments. (B) 1, representative casein zymogram. Density-arrested LU cells were either infected with HCMV (5 PFU/cell) or mock infected. The cells were harvested at the times indicated and processed for zymography as described in Materials and Methods. Since calpain activity is pH dependent, the gels were incubated in incubation buffer at either pH 7.5 (optimal for μ-calpain) or pH 7.3 (optimal for m-calpain). Purified μ- and m-calpain (0.1 U of each) were added to the sample in lane 1 as a positive control. The upper band in each lane corresponds to μ-calpain, whereas the lower band corresponds to m-calpain (89). 2, data for calpain activity based on the means (± standard deviation) of the intensity of bands at each time relative to the intensity of the corresponding band at 0 h p.i. for the zymograms shown above (◆, μ-calpain, HCMV-infected cells; ◇, μ-calpain, mock-infected cells; ●, m-calpain, HCMV-infected cells; ○, m-calpain, mock-infected cells). (C) 1 to 3, abundances of m-calpain, μ-calpain, and calpastatin in parallel cell cultures as determined by Western blot analysis (results of a representative experiment); 4, densitometric analysis of the results shown in panel A and two additional experiments with standard deviation. Solid symbols (◆, m-calpain; ●, μ-calpain, ▲, calpastatin) and line represent the means of the results for HCMV-infected cells; open symbols (◇, m-calpain; ○, μ-calpain; △, calpastatin) and dashed line represent the results for mock-infected cells. Error bars not shown are smaller than the symbol.

synthesis. Data from this laboratory (19) and others (45) have demonstrated that HCMV infection substantially increases E kinase activity in arrested cells. In density-arrested cells, E kinase activity is first substantially increased at 24 h p.i., reaches its maximum level by 72 h, and is still detectable at 96 h (19), well into the late phase of HCMV replication.

Several mechanisms have been identified that regulate the activity of E kinase. For example, an increased cellular abundance of cyclin E is associated with activation of E kinase (reference 65 and references therein). HCMV infection has been shown to transcriptionally activate the expression of cyclin E, but the time course for the increase in E kinase activity is delayed relative to the changes in the cellular abundance of cyclin E (19). The cellular levels of cyclin E protein in density-arrested cells increase substantially by 12 h p.i., peak by 24 h, and are in decline by 48 h (19). Accordingly, not only does activation of E kinase lag behind the increase in cyclin E abundance, but E kinase remains activated longer than would be anticipated based solely on cyclin E abundance (19). For example, despite the decrease in cyclin E levels at 48 h and continuing reduction thereafter, E kinase activity increases remarkably at 48 h p.i., peaks at 72 h, and still remains high at 96 h p.i. The lack of congruity in the data for cyclin E abundance and E kinase activity suggest that other mechanisms are involved in the regulation of E kinase activity in HCMV-infected cells.

The activity of cyclin E-Cdk2 complexes is also regulated by CKIs, including $p21^{cip1}$. In fact, it has been suggested that $p21^{cip1}$ establishes a threshold in the nucleus (34, 44, 75, 86), which must be exceeded if E kinase activity is to develop from cyclin E-Cdk2 complexes. Increases in E kinase activity in HCMV-infected, density-arrested cells closely parallel a substantial reduction in the abundance of $p21^{cip1}$ by 24 h p.i. (19). Our earlier results suggest that the delay in activation of E kinase activity after the increase in cyclin E abundance and the persistence of the cyclin E-associated kinase activity after cyclin E levels are sharply reduced are related to the effects of the CKIs $p21^{cip1}$ and $p27^{kip1}$ (19). Both $p21^{cip1}$ and $p27^{kip1}$ are abundant in quiescent LU cells, contributing to the observed delay between increasing cyclin E levels and activation of E kinase until cyclin E-Cdk2 complexes exceed the threshold established by $p21^{cip1}$ and $p27^{kip1}$. Later, as cyclin E levels are falling, E kinase remains active because HCMV infection stimulates reduction of both $p21^{cip1}$ and $p27^{kip1}$, particularly of $p21^{cip1}$ (19). Thus, the initial phase of activation of E kinase activity is associated with a substantial increase in cylin E levels and decrease in $p21^{cip1}$ levels, while a substantial and sustained decrease in $p21^{cip1}$ is associated with the persistence of E kinase activity observed during HCMV infection (Fig. 1D).

Proteolysis of $p21^{cip1}$ in mock- and HCMV-infected cells. The abundance of each protein in a living cell depends on the balance between its synthesis and degradation, and therefore intracellular proteolysis is of critical importance in maintaining functional concentrations. In fact, many regulatory proteins are subject to proteolytic degradation, which allows cells to rapidly adjust their concentration. Reduction of $p21^{cip1}$ levels in noninfected cells is reported to be mediated by ubiquitination and proteasome degradation (10, 35, 38, 85). Indeed, in mock-infected LU cells, proteasome degradation was the predominant pathway for $p21^{cip1}$ proteolysis (Fig. 2). In contrast, the present results indicate that in HCMV-infected cells, the proteosome is responsible for little, whereas calpain activity is responsible for much, of the degradation of $p21^{cip1}$ observed during the progression of the virus infection. The early decrease in $p21^{cip1}$ abundance appears to be related in part to decreasing RNA levels (Fig. 1) and in part to increasing calpain-mediated degradation (Fig. 6). Since the half-life of $p21^{cip1}$ is reported to be approximately 2 h (46), a decrease in RNA levels would predictably result in lower levels of the protein. When the activity of calpain was inhibited at these early times (e.g., 9 h p.i.), $p21^{cip1}$ levels rose to 158% of the preinfection levels, compared with 83.1% in the absence of calpain inhibitors (Fig. 6). At 24 h p.i., when a substantial increase in E kinase activity is first detected, $p21^{cip1}$ levels are at 23% of their preinfection levels, but when calpain activity is blocked, $p21^{cip1}$ levels are at 85% of their preinfection levels. This level of $p21^{cip1}$ is similar to the levels observed in absence of calpain inhibition at 9 and 12 h p.i. (83 and 72%, respectively), when E kinase activity is at a very low level (19). Thus, calpain-mediated mechanisms appear to be responsible for the degradation of a substantial portion of $p21^{cip1}$, even at early times in HCMV replication. At later times (after 48 h), when RNA levels are relatively constant, calpain-mediated mechanisms are largely responsible for the continuing decrease in $p21^{cip1}$ abundance. During this period (48 and 72 h p.i.), inhibition of calpain-catalyzed proteolysis produced cellular levels of $p21^{cip1}$ that were from 77.5 to 36.4% of the preinfection levels, whereas in the absence of calpain inhibitors, the level of $p21^{cip1}$ fell to 10.8 and 6.2%, respectively, of the preinfection level (Fig. 6). Thus, HCMV-induced activation of calpain is important at both early and late times in controlling the abundance of $p21^{cip1}$ and, accordingly, the activity of the cyclin E-associated kinase. The calpain effect seems particularly noteworthy in extending the activation of E kinase well into the late phase of HCMV replication. Since E kinase activity has been shown to be necessary for high infectious yields of HCMV (18), it is tempting to speculate that the sustained calpain activity and persistent low levels of $p21^{cip1}$ support durable activation of E kinase and production of high yields of infectious HCMV. Indeed, preliminary experiments indicate that a single exposure to Z-Leu-Leu-H (100 μM) is sufficient to reduce HCMV infectious yields at 96 h PI by 99.7% ($P < 0.05$).

Several experimental approaches support the view that calpain activity and $p21^{cip1}$ levels are closely coupled in HCMV-infected cells. Calpain activity increases in a temporal pattern that is consistent with the time courses for the decrease in $p21^{cip1}$ abundance and the increase in E kinase activity in HCMV-infected cells (compare Fig. 7 with Fig. 1; see also reference 19). The time course for E64d protection of $p21^{cip1}$ (Fig. 3) closely parallels the decrease in $p21^{cip1}$ (Fig. 1) and the increase in calpain activity (Fig. 7). The presence of inhibitors of calpain activity (E64d, Z-Leu-Leu-H) protects up to about 80% of $p21^{cip1}$ (Fig. 4 and 5). The concentration effect of E64d in providing protection of $p21^{cip1}$ in HCMV-infected LU cells is in the range (6.25 to 100 μM) observed in earlier studies to protect other targets of calpain-mediated proteolysis (54). In cell-free studies, purified calpain degrades purified recombinant $p21^{cip1}$ (Fig. 8). Considered together, these results indicate that calpain activity is largely responsible for the substantial decrease in $p21^{cip1}$ observed during HCMV infection.

ecules in the limited cell cycle progression induced by productive HCMV infection.

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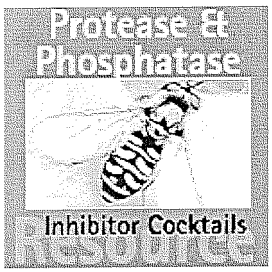

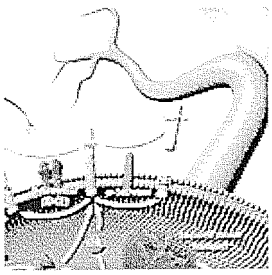

EST

Cat. No. 330005

Calcium Signaling Tools » Calpains, Calpain Inhibitors, and Calpain Substrate

E-64d
(2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane Ethyl Ester
Loxistatin

White solid. A cell-permeable, irreversible inhibitor of cysteine proteases. Similar to E-64 (Cat. No. 324890) but devoid of charged groups. Reported to inhibit calpain-1 activation. The inhibitory activity of EST has been attributed to E-64c, the free acid formed by hydrolysis of the ester *in vivo*. Used in animal models of muscular dystrophy. *Purity:* ≥*98% by TLC.* RTECS RR0404300, CAS 88321-09-9.

Ref.: Inomata, M., et al. 1996. *Arch. Biochem. Biophys.* **328,** 129. Mehdi, S. 1991. *Trends Biochem. Sci.* **16,** 150. McGowan, E.B., et al. 1989. *Biochem. Biophys. Res. Commun.* **158,** 432. Tamai, M., et al. 1987. *J. Pharmacobiodyn.* **10,** 678. Komatsu, K., et al. 1986. *Exp. Neurol.* **91,** 23. Tamai, M., et al. 1986. *J. Pharmacobiodyn.* **9,** 672.
R: 36/37/38; S: 26-36

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Size	In Stock	Qty	Price
1 mg	Y		$100

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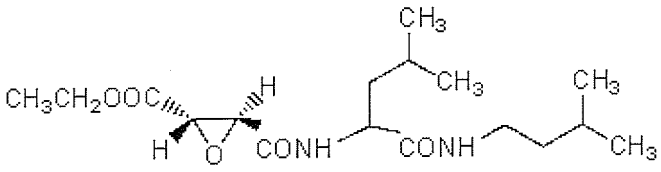
* NOTE: In Stock status is based on item availability worldwide.

Solubility	Molecular Formula	Mol. Wt.
$CHCl_3$ or EtOH	$C_{17}H_{30}N_2O_5$	342.4



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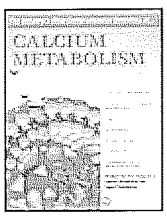
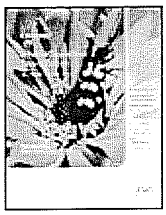
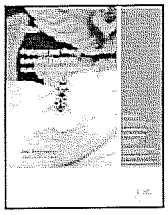


Calcium Metabolism and Related Products Brochure


Inhibitor Sourcebook


Protease and Phosphatase Inhibitor Cocktails Brochure

PEPTIDES INTERNATIONAL

The ENZYME INHIBITOR Guide

Some inhibitors may permeate some cell systems and not others.
* Inhibitors can be permeable in some cases (particularly with aldehyde-based inhibitors), but may require greater concentrations and longer duration.
▲ Able to permeate some cells according to several references.

CODE	PRODUCT	TARGET	WORKING CONC. GUIDE	SOLUBILITY	CELL PERMEABLE #	REVERSIBLE	QTY	USD
IAR-4148-v	**Arphamenine A**	Aminopeptidase B	100 - 500 nM	Distilled water		YES	0.5 mg	34
IAR-4149-v	**Arphamenine B**	Aminopeptidase B	50 - 100 nM	Distilled water		YES	0.5 mg	39
IBS-4093-PI	**Ubenimex (Bestatin)**	Aminopeptidase B and Leucine Aminopeptidase	1-10 µM IC_{50} = 4 µM	Acetone, DMSO	YES	YES	25 mg	142
ILH-4249-v	**Leuhistin**	Aminopeptidase M	0.1 - 3 µM Ki = 230 nM (aminopeptidase M)	Distilled water		YES	0.5 mg	36
IAC-4157-v	**Actinonin**	Aminopeptidase M and Leucyl Aminopeptidase	1 - 200 µM	Acetic Acid		YES	0.5 mg	34
IPH-4342-v	**Phebestin**	Aminopeptidase N	Ki = 47 µM	DMSO			5 mg	100
IAM-4095-v	**Amastatin**	Aminopeptidase A and Leucyl Aminopeptidase	1 - 10 µM	DMSO		YES	0.5 mg	69
IDP-4132-v	**Diprotin A**	Dipeptidyl Aminopeptidase IV	10 - 50 µM Ki = 2.2 µM	Distilled water		YES	0.5 mg	18
IEB-4155	**Ebelactone A**	N-Formylmethionyl Aminopeptidase, Lipase, and Esterase	1 - 10 µM	MeOH and EtOH	NO*	YES	25 mg	995
IEB-4156	**Ebelactone B**	N-Formylmethionyl Aminopeptidase, Lipase, and Esterase	1 - 10 µM	MeOH and EtOH	NO*	YES	25 mg	995
IAB-4009-v	**Bradykinin-Potenitator B**	Angiotensin I Converting Enzyme, Peptidyl-dipeptidase A, Kinase II	IC_{50} = 1.1 µM	Distilled water			0.5 mg	30
IAC-4010-v	**Bradykinin-Potenitator C**	Angiotensin I Converting Enzyme, Peptidyl-dipeptidase A, Kinase II	IC_{50} = 7.1 µM	Distilled water			0.5 mg	23
IBK-4097-v	**Des-Pro²-Bradykinin**	Angiotensin I Converting Enzyme, Peptidyl-dipeptidase A, Kinase II	10 µM	Distilled water			0.5 mg	30
IFR-4190-v	**Foroxymithine**	Angiotensin I Converting Enzyme	IC_{50} = 12 µM	Distilled water			0.5 mg	65
IRG-4395-v	**KYT-1**	Arg-Gingipain	1 - 100 µM	DMSO			1 mg	263
IKG-4396-v	**KYT-36**	Arg-Gingipain	1 - 100 µM	DMSO			1 mg	263
IKK-4374-v	**Lys-Lys-Lys-Leu-Arg-Arg-Gln-Glu-Ala-Phe-Asp-Ala-Tyr (KKKLRRQEAFDAY or AIP)**	Calmodulin-Dependent Protein Kinase II	IC_{50} = 4 nM	Distilled water			0.5 mg	100
IZL-3178-v	**Z-Leu-Leu-H (aldehyde) Z-LL-CHO**	Calpain	10 - 100 µM	DMSO	YES	YES	5 mg	40
IES-4096-v	**E-64**	Calpain, Thiol Proteases	1-10 µM	Distilled water	NO*▲ (Can Permeate SF9 Insect Cells)	NO	0.5 mg	37
IEC-4320-v	**E-64-c**	Calpain and Cathepsin B/H/L, Thiol Proteases	1-10 µM	DMSO	NO*	NO	5 mg	100

(continued inside)

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Some inhibitors may permeate some cell systems and not others.
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▲ Able to permeate some cells according to several references.

CODE	PRODUCT	TARGET	WORKING CONC. GUIDE	SOLUBILITY	CELL PERMEABLE #	REVERSIBLE	QTY	USD
IEC-4321-v	**E-64-d**	Calpain and Cathepsin B/H/L, Thiol Proteases	1-10 µM	DMSO	YES	NO	5 mg	100
ICA-3187-v	**Ac-Trp-Glu-His-Asp-H (aldehyde) [Ac-WEHD-CHO]**	Caspase-1	10 - 100 µM	DMSO	NO*	YES	5 mg	200
IAT-3165-v	**Ac-Tyr-Val-Ala-Asp-H (aldehyde) [Ac-YVAD-CHO]**	Caspase-1/4	10 - 100 µM	DMSO	NO*	YES	5 mg	195
IAT-3166-v	**Ac-Tyr-Val-Lys-Asp-H (aldehyde) Ac-YVKD-CHO**	Caspase-1	10 - 100 µM Ki = 0.038 µM	DMSO	NO*	YES	5 mg	195
IVD-3204-v	**Ac-Val-Asp-Val-Ala-Asp-H (aldehyde) Ac-VDVAD-CHO**	Caspase-2	0.0001 - 100 µM Ki = 3.5 nM	DMSO	NO*	YES	5 mg	300
IAC-3221-v	**Ac-Asp-Asn-Leu-Asp-H (aldehyde) Ac-DNLD-CHO**	Caspase-3	1 - 30 nM IC_{50} = 3.2 nM	DMSO	NO*	YES	5 mg	212
ICA-3192-v	**Ac-Asp-Met-Gln-Asp-H (aldehyde) Ac-DMQD-CHO**	Caspase-3	10 - 100 µM	DMSO	NO*▲	YES	5 mg	300
IAP-3172-v	**Ac-Asp-Glu-Val-Asp-H (aldehyde) Ac-DEVD-CHO**	Caspase-3/7/8	1 - 100 µM	DMSO	NO*▲	YES	5 mg	195
IBA-3173-v	**Biotinyl-Asp-Glu-Val-Asp-H (aldehyde) Biotinyl-DEVD-CHO**	Caspase-3/7/8	10 - 100 µM	DMSO	NO*	YES	1 mg	99
IVA-3182-v	**Ac-Val-Glu-Ile-Asp-H (aldehyde) Ac-VEID-CHO**	Caspase-6	10 - 100 µM	DMSO	NO*	YES	5 mg	218
ICA-3194-v	**Ac-Asp-Gln-Thr-Asp-H (aldehyde) Ac-DQTD-CHO**	Caspase-7/3	50 - 100 µM	DMSO	NO*▲	YES	5 mg	250
ICA-3196-v	**Ac-Ile-Glu-Thr-Asp-H (aldehyde) Ac-IETD-CHO**	Caspase-8/6, Granzyme B	50 - 100 µM	DMSO	NO*	YES	5 mg	200
ICA-3199-v	**Ac-Leu-Glu-His-Asp-H (aldehyde) Ac-LEHD-CHO**	Caspase-9	0.01 - 10 µM	DMSO	NO*	YES	5 mg	250
ICL-3180-v	**Ac-Tyr-Val-Ala-Asp-CH_2Cl Ac-YVAD-CMK**	Caspases	1 - 200 µM IC_{50} ~ 50 µM	DMSO	YES	NO	5 mg	200
ICE-3174-v	**Z-Asp-CH_2-DCB**	Caspases	50 - 200 µM	DMSO	YES	NO	5 mg	195
ICA-3189-v	**Z-Glu-Lys(Biotinyl)-Asp-CH_2-DMB [Z-EK(bio)D-aomk]**	Caspases	1 µM	DMSO			1 mg	100
ICA-3188-v	**Z-Val-Ala-Asp(OMe)-CH_2F [Z-VAD(OMe)-FMK]**	Caspases	0.05 - 100 µM	DMSO	YES	NO	1 mg	100
IEC-3644-PI	**N-(4-Biphenylacetyl)-Cys(Me)-D-Arg-Phe-N-Phenylethylamide** (Cathepsin L, Inhibitor VI)	Cathepsin L	Ki = 19 nM (Cathepsin L)			YES	5 mg	65
IPA-4397	**Pepstatin A (Purity > 90% by HPLC)**	Cathepsin D/E, Aspartyl proteases, Pepsin, Renin	1 - 10 µM	DMSO, Ethanol,or Acetic Acid (9:1 v/v)	NO*▲	YES (Can Permeate SF9 Insect Cells)	25 mg	75
IAL-3678-PI	**Ac-Leu-Leu-Met-H (aldehyde) [ALLM]**	Cathepsin B/L, Calpain I/II, Proteasome (weak)	0.1 - 250 µM	DMSO, MeOH, and EtOH	YES	YES	5 mg	46

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Annals of the New York Academy of Sciences 939:436-449 (2001)

Cell Death in Spinal Cord Injury (SCI) Requires *de Novo* Protein Synthesis

Calpain Inhibitor E-64-d Provides Neuroprotection in SCI Lesion and Penumbra

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Degradation of cytoskeletal proteins by calpain, a Ca^{2+}-dependent cysteine protease, may promote neuronal apoptosis in the lesion and surrounding areas following spinal cord injury (SCI). Clinically relevant moderate (40 g-cm force) SCI in rats was induced at T12 by a standardized weight-drop method. Internucleosomal DNA fragmentation or apoptosis in the lesion was inhibited by 24-h treatment of SCI rats with cycloheximide (1 mg/kg), indicating a requirement for *de novo* protein synthesis in this process. To prove an involvement of calpain activity in mediation of apoptosis in SCI, we treated SCI rats with a cell-permeable calpain inhibitor E-64-d (1 mg/kg). Following 24-h treatment, a 5-cm-long spinal cord section centered at the lesion was collected, and divided equally into five segments (1 cm each) to determine calpain activity, as shown by degradation of the 68-kD neurofilament protein (NFP), and apoptosis as indicated by internucleosomal DNA fragmentation. Neurodegeneration propagated from the site of injury to neighboring rostral and caudal regions. Both calpain activity and apoptosis were readily detectable in the lesion, and moderately so in neighboring areas of untreated SCI rats, whereas these were almost undetectable in E-64-d-treated SCI rats, and absent in sham animals. Results indicate that apoptosis in the SCI lesion and penumbra is prominently associated with calpain activity and is inhibited by the calpain inhibitor E-64-d providing neuroprotective benefit.

Key Words: Cell death • Spinal cord injury • Calpain inhibitor • Neuroprotection